AD_____

Award Number: W81XWH-08-1-0391

TITLE: SLC5A8-mediated switching of STAT3 from a pro-oncogenic signal into a pro-apoptotic signal in breast cancer

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REPORT DATE: June 2009

TYPE OF REPORT: Revised Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

*Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2009	Revised Annual	1 June 2008 – 31 May 2009

4. TITLE AND SUBTITLE		5a. CONTRACT NUMBER
SLC5A8-mediated switching of STAT3 from a pro-oncogenic signal into a pro-apoptotic signal in breast cancer		5b. GRANT NUMBER W81XWH-08-1-0391
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)		5d. PROJECT NUMBER
Muthusamy Thangaraju, Ph.D.		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Medical College of Georgia Research Institute Augusta, GA 30912	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this study is to establish the functional significance of SLC5A8, a butyrate transporter and tumor suppressor, in STAT3 associated cellular apoptosis. In normal mammary epithelium both STAT3 and SLC5A8 are active and functional and thus it maintains the cellular homeostasis by regulation of cellular apoptosis. However, many cancers including breast cancer is associated with constitutively active STAT3 with inactive SLC5A8 expression.Either the functional co-operation between the STAT3 and SLC5A8 in normal mammary epithelium or the functional redundancy between STAT3 and SLC5A8 in human breast cancer is not known. In order to understand the functional implication of SLC5A8 in STAT3 associated cellular apoptosis and pro-oncogenic potential in human mammary epithelium, we have developed a stable cell lines in human normal mammary epithelial cell line that express the functional STAT3 and SLC5A8, HMEC and MCF10A, with constitutively active STAT3 (STAT3C). Further, we have developed a stable cell line in these cells with SLC5A8shRNA. To check the functional importance of SLC5A8 in STAT3 associated apoptosis, we have developed a stable cell lines in human breast cancer cell line MCF7, which constitutively express STAT3 with undetectable expression of SLC5A8, with functional SLC5A8. We are using these stable cell lines to delineate the molecular signaling involved in regulation of STAT3 and SLC5A8 in human mammary epithelium.

15. SUBJECT TERMS
Breast Cancer, STAT3, Constitutively active STAT3 (STAT3), SLC5A8, Human mammary epithelial cells, HMEC, MCF10A, Human breast cancer cells, MCF7

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION: The purpose of this study is to establish the functional significance of SLC5A8, a butyrate transporter and tumor suppressor, in STAT3 associated cellular apoptosis. In normal mammary epithelium both STAT3 and SLC5A8 are active and functional and thus it maintains the cellular homeostasis by regulation of cellular apoptosis. However, many cancers including breast cancer is associated with constitutively active STAT3 with inactive SLC5A8 expression. Either the functional co-operation between the STAT3 and SLC5A8 in normal mammary epithelium or the functional redundancy between STAT3 and SLC5A8 in human breast cancer is not known. In order to understand the functional implication of SLC5A8 in STAT3 associated cellular apoptosis and pro-oncogenic potential in human mammary epithelium, we have developed a stable cell lines in human normal mammary epithelial cell line that express the functional STAT3 and SLC5A8, HMEC and MCF10A, with constitutively active STAT3 (STAT3C). Further, we have developed a stable cell line in these cells with SLC5A8shRNA. To check the functional importance of SLC5A8 in STAT3 associated apoptosis, we have developed a stable cell lines in human breast cancer cell line MCF7, which constitutively express STAT3 with undetectable expression of SLC5A8, with functional SLC5A8. We are using these stable cell lines to delineate the molecular signaling involved in regulation of STAT3 and SLC5A8 in human mammary epithelium.

2. BODY:

Statement of Work

SLC5A8, a molecular switch for STAT3 induced pro-oncogenic to pro-apoptotic signals

| Specific aim	Year 1	Year 2	Year 3
Aim 1A	X (done)	X	
Aim 1B	X (done)	X	
Aim 1C	X (done)	X	
Aim 1D	X (done)	X	X
Aim 2A		X	X
Aim 2B		X	X
Aim 2C		X	X

Specific Aim 1:

Task 1: Expression of constitutively active STAT3 in human mammary epithelial cells

a.   Develop stable clones that constitutively express STAT3 (STAT3C) in human normal mammary epithelial cell line HMEC and in human breast adenocarcinoma cell line MCF7.

Background and Significance: Our preliminary studies have shown that activation of Stat3 during mammary gland involution occurs concurrently with increased slc5a8 expression in normal mammary epithelial cells. However, in the breast cancer constitutive activation of STAT3 is associated with loss of function of SLC5A8 and we do not know if these two events have a direct cause-and-effect relationship. We wanted to test these observations by directly assessing the influence of activated STAT3 on SLC5A8 expression in human normal mammary cells like HMEC and MCF10A.

Thus, we have developed stable clones of constitutively active STAT3 (STAT3C) in two human normal mammary epithelial cell lines (HMEC and MCF10A) by using the STAT3C, constitutively active STAT3, construct. To make these stable clones, we have transfected the STAT3C in HMEC and MCF10A cells and selected the colonies with 100 g/ml G418 for 4 weeks. After 4 weeks, independent colonies were picked-up and expanded. To check the stable expression of STAT3C in these cells, RNA and proteins were extracted and the expression of STAT3 mRNA was analyzed by northern blotting using the p32 labeled STAT3 specific probe (Fig.1). To check the constitutively expressed STAT3C in HMEC and MCF10A stable clones, proteins were extracted in two vector (pcDNA3.1) and three STAT3C (Clone #1, 2, and 3) clones and resolved in 8% SDS-PAGE gels. Proteins were transferred into to the nitrocellulose membrane and hybridized with the phosphorylated STAT3, pY-STAT3, and basal STAT3

Fig. 1: Stable expression of constutively acitve STAT3 (STAT3C) in human mammary epithelial cell lines: HMEC and MCF10A were stable transfected with pcDNA3.1 and STATC. After 4 weeks of G418 (100μg/ml) selection, individual clones were picked-up and expanded. RNA and proteins were extracted from these stable clones and the expression of STAT3 mRNA expression were analyzed by Northern bloting and the constitutively active STAT3 were analyzed by Western blotting using phospho-STAT3 antibody.

antibodies (Fig. 1). We will use these stable clones to check the methylation status of SLC5A8 as well as will use to test the tumorigenic potential of STAT3C by using the mouse xenograft.
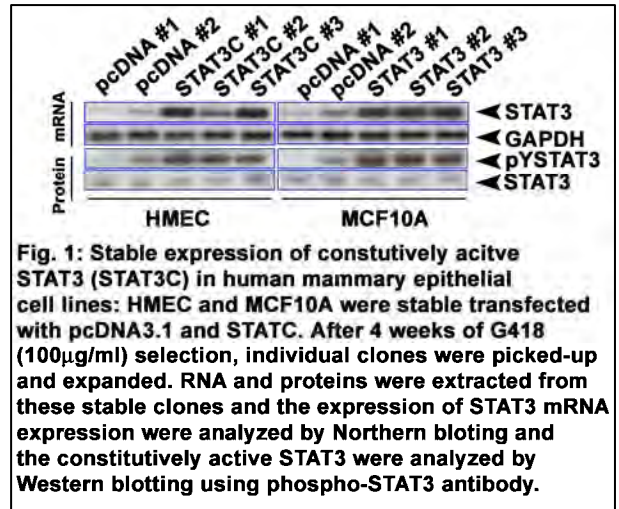
b.   Using these stable clones, we will perform the i*n vitro* functional assays to characterize the role of STAT3 and its constitutive active form (STAT3C) in normal mammary epithelial as well as in breast tumor epithelial cells.

We have used these stable clones to check whether the constitutively active STAT3C involved in regulation of cellular transformation by inactivation of SLC5A8 in normal human mammary epithelial cells. (1) We have extracted the RNA and protein samples from the stable clones and checked the expression of SLC5A8 by quantitative RT-PCR and western blotting using the SLC5A8 specific antibody. (2) To check the functional activity of SLC5A8, we have measured Na^+-dependent uptake of nicotinate using these stable cell lines. (3) To check the constitutively active STAT3 associated cellular transformation, we have done the anchroach independent colony formation assay, Giemsa staining colony assay and MTT assay. (4) Further to check the status of PI3K activity in vector and STAT3C expressed stable cell lines, we have analyzed the expression of PI3K isoforms p85α, p55α, and p50α by quantitative RT-PCR. (5) We have also analyzed the protein expression of all these three isoforms of PI3K, p85α, p55α, and p50α, by western blotting by using the subunit specific antibodies.
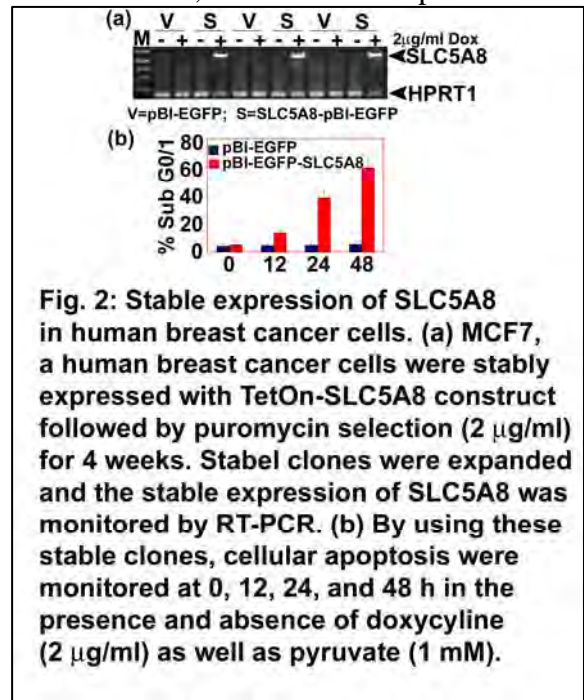
c.   In order to establish the *in vivo* functional consequences of STAT3wt and its constitutive active form (STAT3C) in tumor formation, we will use the above mentioned stable clones to perform the mouse xenogranft.

To check the in vivo functional role of constitutively active STAT3, we are in the process of doing the mouse xenograft using these stable clones.

Task 2: Generation of stable MCF7 cell lines expressing SLC5A8

a. Develop stable clones with SLC4A8 in human breast cancer cell line (MCF7) by using the TetOn regulated system.

To check the tumor suppressor function of SLC5A8 in human breast cancer, we have developed a controlled tetracycline regulated TetOn system regulated expression of SLC5A8 in human breast cancer cell line MCF7. To check the stable expression of SLC5A8 in MCF7 cells, we have cloned the SLC5A8 in Tetracyclin responsive element as well as enhanced green fluorescence protein (EGFP) containing pCMV promoter driven pBI-EGFP vector. The stable MCF7-TetOn cells were purchased from Clonetics and transfected the SLC5A8-pBI-EGFP and pBaBe-Puro plasmids in this TetOn regulated cell line. After 4 weeks of puromycin selection (2 µg/ml) independent colonies were picked-up and expanded for the further analysis. RNA and protein were extracted from the independent clones and checked the expression of SLC5A8 (Fig. 2). We have used these stable clones to check the functional activity of SLC5A8 by using the Na+ dependent Nicotinate uptake. We have also used these stable clones to check the functional importance of SLC5A8 in regulation of SLC5A8 ligand associated cellular apoptosis in human breast cancer cells. To do this, we have incubated the vector controlled as well as SLC5A8 expressed stable clones in the presence and

Fig. 2: Stable expression of SLC5A8 in human breast cancer cells. (a) MCF7, a human breast cancer cells were stably expressed with TetOn-SLC5A8 construct followed by puromycin selection (2 µg/ml) for 4 weeks. Stabel clones were expanded and the stable expression of SLC5A8 was monitored by RT-PCR. (b) By using these stable clones, cellular apoptosis were monitored at 0, 12, 24, and 48 h in the presence and absence of doxycyline (2 µg/ml) as well as pyruvate (1 mM).

absence of doxycyclin, a tetracycline analog, (2 µg/ml) and the sodium pyruvate (1mM) for 0, 12, 24, and 48 h. Cells were harvested and processed for the propidium iodide stained cell cycle analysis. The sub G1 populations, SubG0/1, were quantified and plotted (Fig. 2). Values are represented as a Mean and SEM of four independent experiments.

b.   Using these stable clones, we will perform the *in vitro* functional assays to establish the role of SLC5A8 in human breast cancer cells.

We are using these stable clones to check the functional role of SLC5A8 in tumor suppression or prevention of SLC5A8 in mammary tumorigenesis *in vivo*. To do this we are using the mouse xenograft and the studies are in progress now.

c.   *In vivo* functional characterization of these stable clones using nude mice.
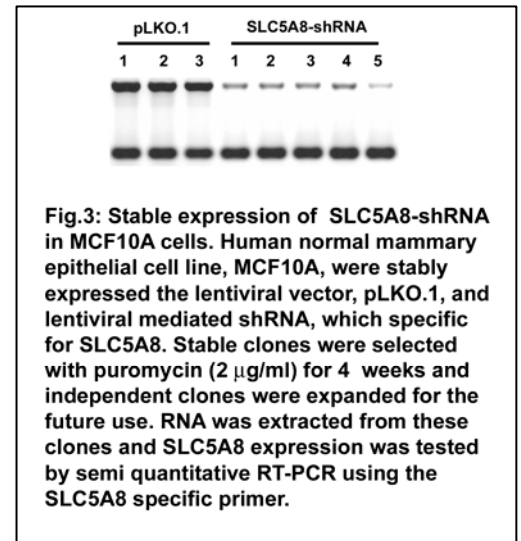
We will use the mouse xenograft tumor samples of vector as well as SLC5A8 stable clones to check the differential regulation of tumor suppressor as well as oncogenes in these samples.

Task 3: Generation of stable MCF10A cell lines expressing SLC5A8 siRNA

a.   Develop stable clones in human mammary epithelial cell line (MCF10A) with SLC4A8 siRNA.

Background and Significance: HMEC and MCF10A cells are normal mammary epithelial cells. We expect that activation of STAT3 in these cells by stretching or by treatment with leukemia inhibitory factor (LIF) will lead to apoptosis. We hypothesize that cell death would occur in these cells in the presence of activated STAT3 because of the functional expression of SLC5A8. However, either mechanical stretching or LIF treatment unable to induces apoptosis in cells that are inactive for SLC5A8. To test this possibility, we wanted to generate MCF10A cell lines in which SLC5A8 is silenced by siRNA and then subject the cells to stretching- or LIF-induced STAT3 activation to see if there is any difference in cell death between SLC5A8-expressing cells and SLC5A8-deficient cells.

Thus, we have developed a stable expression of SLC5A8shRNA in human normal mammary epithelial cell line (MCF10A). To develop this stable cell line, we have stably expressed the lentiviral mediated SLC5A8shRNA in MCF10A cells and the stable clones were selected after 4 weeks of puromycin (2 g/ml) selection. Independent clones were picked-up and expanded. To check the efficiencies of the shRNA in inactivation of SLC5A8, we have harvested the cells from three independent clones of scrambled shRNA (pLKO.1) and five independent clones of SLC5A8shRNA (Clones 1-5) and extracted RNA from these cells and cDNA were synthesized by using the RT-PCR kit. Using the human specific SLC5A8 primer, we have checked the expression of SLC5A8 in pLKO.1 and SLC5A8shRNA stable clones (Fig. 3). We will use these stable clones to (1) to characterize role of SLC5A8 in regulation of tumor associated genes and or proteins and (2) to establish the functional significance of SLC5A8 in tumor prevention or tumor formation, we will perform the *in vivo* assay using the mouse xenogranfts.

Fig.3: Stable expression of SLC5A8-shRNA in MCF10A cells. Human normal mammary epithelial cell line, MCF10A, were stably expressed the lentiviral vector, pLKO.1, and lentiviral mediated shRNA, which specific for SLC5A8. Stable clones were selected with puromycin (2 μg/ml) for 4 weeks and independent clones were expanded for the future use. RNA was extracted from these clones and SLC5A8 expression was tested by semi quantitative RT-PCR using the SLC5A8 specific primer.

3. KEY RESEARCH ACCOMPLISHMENTS: As per the statement of work that proposed in the grant, in the first year of the grant we have generated the following four stable cell lines to test the hypothesis that SLC5A8 is the molecular switch to convert the STAT3 associated pro-oncogenic signal into pro-apoptotic signal in human breast cancer.

Stable Cell line 1: HMEC with constitutively active stable expression of STAT3 (STAT3C).

Stable Cell line 2: MCF10A with constitutively active stable expression of STAT3 (STAT3C).

Stable Cell line 3: MCF7 with stable expression of SLC5A8.

Stable Cell line 4: MCF10A with stable expression of SLC5A8shRNA.

4. REPORTABLE OUTCOMES: We are planning to submit an abstract for the forthcoming AACR conference "**Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications**".

5. CONCLUSION: Now, we have the stable cell lines, which constitutively express STAT3C in human normal mammary epithelial cell lines and a stable cell line, which express the SLC5A8, in human breast cancer cells as well as the stable cell line, which express the SLC5A8shRNA, in human normal mammary epithelial cell lines. We are in the processing of characterizing these stable cell lines to delineate the molecular mechanism involved in regulation of STAT3 and SLC5A8 associated apoptotic signaling. Further, we are in the processing of developing MMTV-HRAS and SLC5A8 transgenic mice for the in vivo studies.

6. REFERENCES: No reference has sited.

7. APPENDICES: No abstract of paper has attached in this report now.

8. SUPPORTING DATA: All supporting data also included in the body of the text.

human mammary epithelial cells. Here, the PI notes that SLC5A8 expression and functional activity have been assessed. In addition, the PI indicates that studies focusing on STAT3C associated cellular transformation has also been conducted and that the expression of various PI3K isoforms has been analyzed in cells expressing STAT3C. With respect to Task 2, the PI has developed stable clones with SLC5A8 in the human breast cancer (MCF7) cell line using the tetracycline regulated TetOn system. Here, the PI cloned SLC5A8 tetracycline responsive element and enhanced green fluorescent protein (EGFP) containing pCMV promoter driven pBI-EGFP vector. SLC5A8-pBI-EGFP and pBaBe-Puro plasmids were subsequently transfected into stable MCF7-TetOn cells. Analyses of RNA and protein extracts from the independent clones validated the expression of SLC5A8. Further, the PI assessed the functional importance of SLC5A8 in the regulation of SLC5A8 ligand associated cellular apoptosis in human breast cancer cells. Here, SLC5A8 stable clones were incubated in the presence or absence of the tetracycline analog doxycyclin (2µg/ml) and sodium pyruvate (1mM) for 0, 12, 24, or 48 hours. Cells were subsequently harvested and processed for propidium iodide stained cell cycle analysis and sub G1 populations (SubG0/1) were quantified. Data from this study indicate that the percentage of SubG0/1 populations was increased at 12, 24, and 48 hours.

In Year 1, the PI has also initiated studies with respect to Task 3. Here, the PI sought to determine whether any differences exist in cell death between SLC5A8-expressing and SLC5A8-deficient (silenced by siRNA) cells by subjecting cells to stretching-or LIF-induced STAT3 activation. To accomplish this, the PI stably expressed SLC5A8shRNA in MCF10A cells. To assess the efficiency of the shRNA in inactivating SLC5A8, the PI harvested cells from independent clones of scrambled shRNA (pLKO.1) and SLC5A8shRNA (clones 1-5). RNA was then extracted from these clones with semi-quantitative RT-PCR revealing that SLC5A8 expression was attenuated in SLC5A8shRNA clones as compared to pLKO.1 clones. Future investigations will characterize the role of SLC5A8 in the regulation of tumor associated genes and/or proteins as well as elucidate the functional significance of SLC5A8 in tumor prevention or formation.

FORMAT/EDITORIAL ISSUES: This first annual report does not conform to USAMRMC reporting requirements.

The PI should note that research milestones, such as completing proposed experiments or generating cell lines, are not acceptable as key research accomplishments. Key research accomplishments should be a bulleted list of key research findings resulting from the achievement of project milestones.

To answer this question, please find the following research accomplishments and related results

RESEARCH ACCOMPLISHMENTS

1. To test the molecular mechanism of SLC5A8 inactivation in human normal mammary epithelial cells, we have also transfected several oncogenes in MCF10A cells and analyzed the SLC5A8 expression and functional activity (Figure 4).

2. Further, to define the role of STAT3C and other oncogenes in SLC5A8 inactivation by regulating the DNA methyl transferases (DNMTs), because DNMTs play a major role in SLC5A8 inactivation , we have tested the DNMTs expression, activity and also constructed a 2.4kb DNMT1 promoter and tested the role of STAT3C and other oncogenes (Figure 5).

These are the two main research accomplishments were made in the first year other than generating several stable cell lines and functional assays. However, we have completed all tasks, which proposed in the grant application as well in the statement of work (SOW). Please see the following SOW for the first year, which proposed in the grant application.

Task 1. Expression of constitutively active STAT3 in human mammary epithelial cells (Months 1-4)

a. Develop stable clones that constitutively express STAT3 (STAT3C) in human normal mammary epithelial cell line HMEC and in human breast adenocarcinoma cell line MCF7.

b. Using these stable clones, we will perform the i*n vitro* functional assays to characterize the role of STAT3 and its constitutive active form (STAT3C) in normal mammary epithelial as well as in breast epithelial cells.

c. In order to establish the *in vivo* functional consequences of STAT3wt and its constitutive active form (STAT3C) in tumor formation, we will use the above mentioned stable clones to perform the mouse xenogranft.

Task 2. Generation of stable MCF7 cell lines expressing SLC5A8 (Months 5-8)

a. Develop stable clones with SLC4A8 in human breast cancer cell line (MCF7) by using the TetOn regulated system.

b. Using these stable clones, we will perform the *in vitro* functional assays to establish the role of SLC5A8 in human breast cancer cells.

c. In vivo functional characterization of these stable clones using nude mice.

Task 3. Generation of stable HMEC cell lines expressing SLC5A8 siRNA (Months 9-12)

a. Develop stable clones in human mammary epithelial cell line (HMEC) with SLC4A8 siRNA.

b. Using these stable clones we will perform the in vitro assay to characterize role of SLC5A8 in regulation of tumor associated genes and or proteins.

c. In order to establish the functional significance of SLC5A8 in tumor prevention or tumor formation, we will perform the in vivo assay using the mouse xenogranfts.
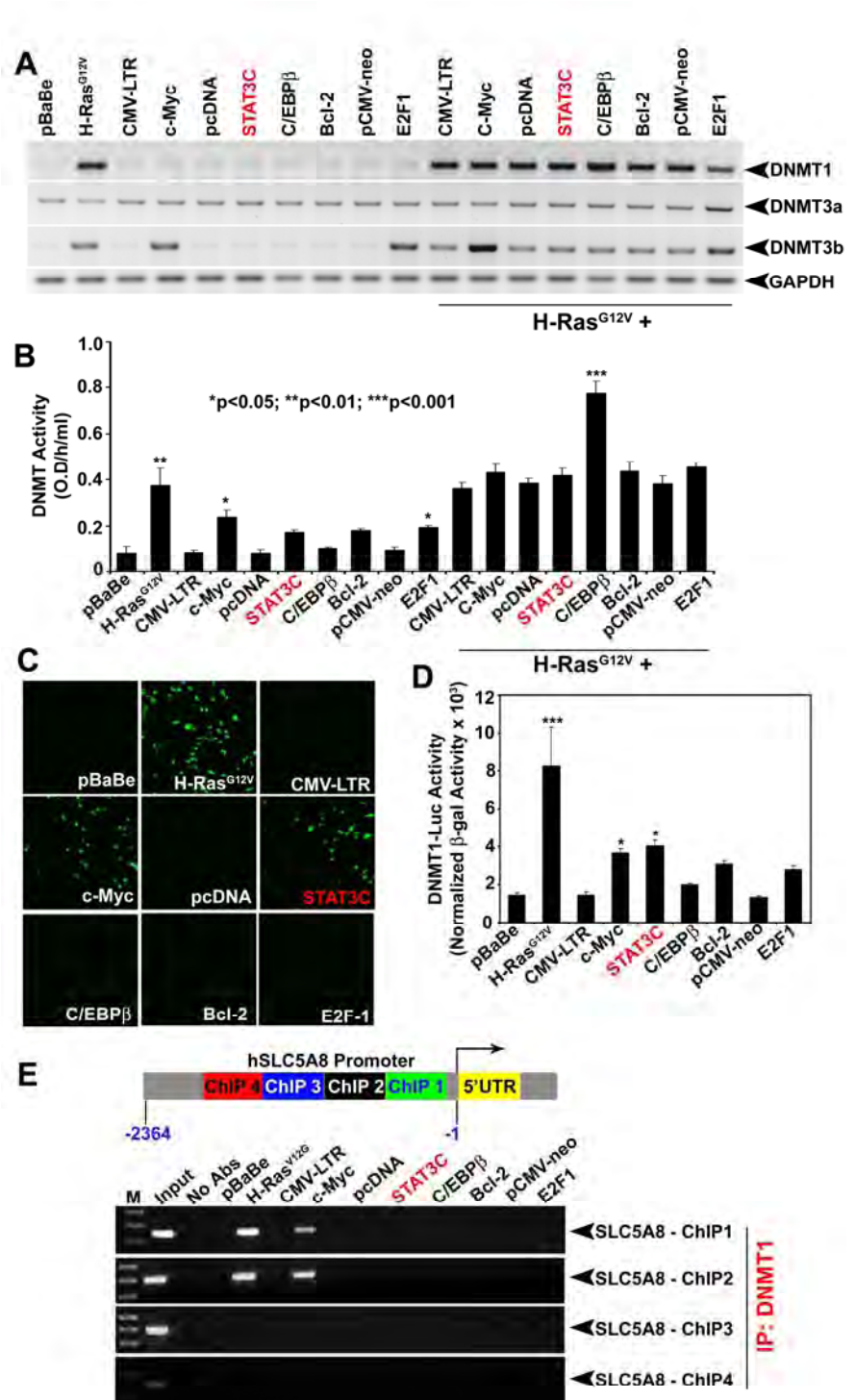
Figure 4: Constitutively active STAT3 (STAT3) play very minor role in SLC5A8 inactivation. The human normal non-transformed mammary epithelial cell line MCF10A was transfected with constitutively active STAT3 (STAT3C) and other oncogenes like HRAS, c-Myc, C/EBPβ, Bcl2 and E2F1. RNA was extracted from these cells and the expression of SLC5A8 was analyzed by semi-quantitative-PCR (**A**) and real-time PCR (**B**). We have also prepared protein lysates from these cells and checked the SLC5A8 protein expression using the SLC5A8 antibody (**C**). We have also tested SLC5A8 function by uptake analysis (**D**). To test whether these oncogenes inactivates SLC5A8 expression by epigenetic mechanism mediated methylation, we prepared the DNA from these cells and treated with bisulfide and then did the methylation specific PCR for SLC5A8 using the methylation specific PCR primers (**E**).
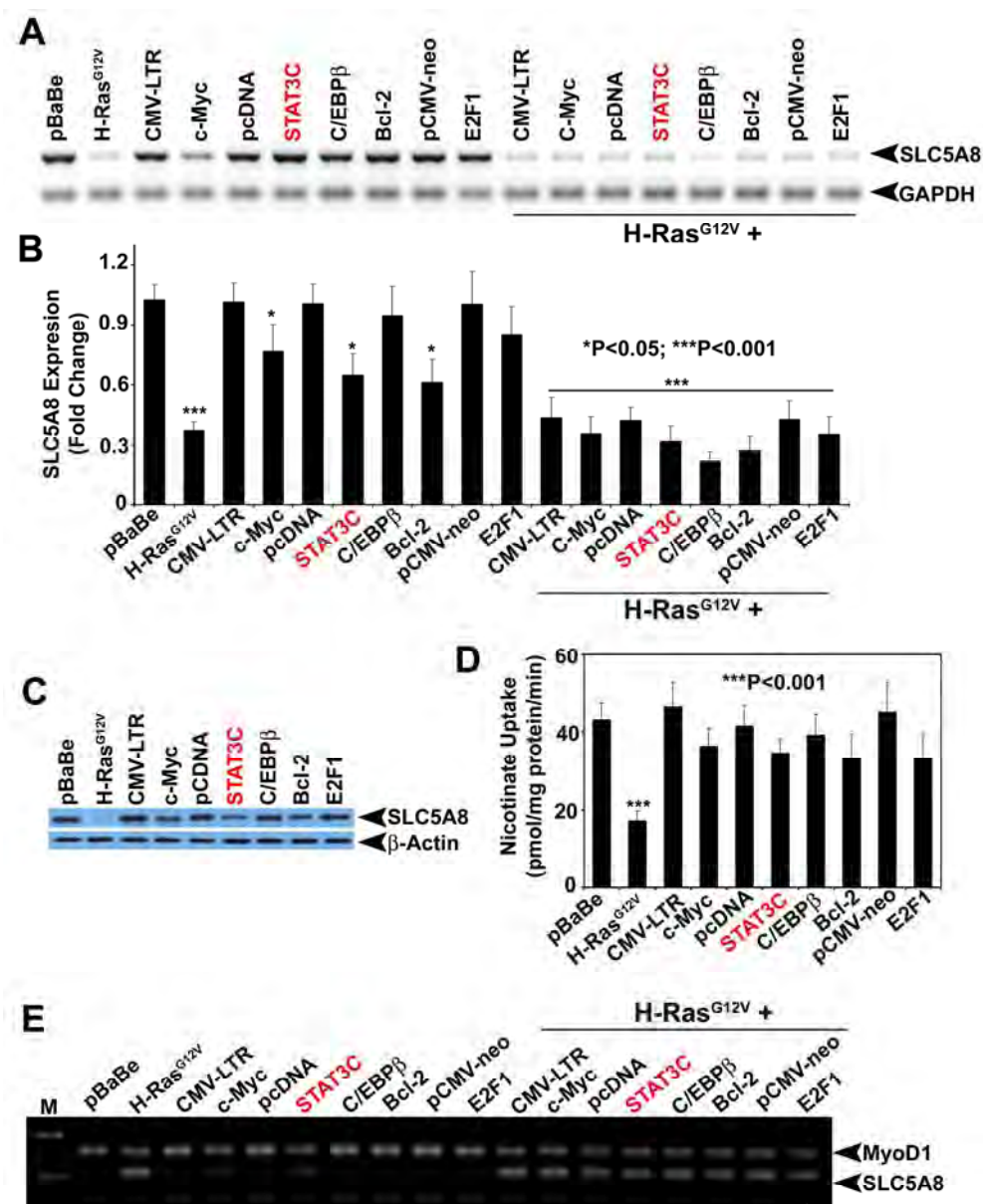
Our data shows that constitutively active STAT3 alone does not involve in SLC5A8 inactivation. However, when combined with oncogenic HRAS, constitutively active STAT3 inactivates SLC5A8 expression by epigenetic mechanism mediated DNA metylation.

Figure 5: Role of Constitutively active STAT3 (STAT3) in regulation of DNA methylation. The human normal non-transformed mammary epithelial cell line MCF10A was transfected with constitutively active STAT3 (STAT3C) and other oncogenes like HRAS, c-Myc, C/EBPβ, Bcl2 and E2F1. RNA was extracted from these cells and the expression of DNA methyltransferses (DNMTs) like DNMT1, 3a and DNMT3b were analyzed by semi-quantitative-PCR (**A**). We have also prepared nuclear extract from these cells and measured the DNMT activity using the commercially available kit (Epigentek, NY) (**B**). We cloned 2.4 kb of human DNMT1 promoter in pTurboGFP-PRL vector as well as in pGL3-Luc vector and then transfected this vector along with STAT3 and other oncogenes into MCF10A cells. After 48h transfection, cells were monitored for GFP fluorescence (**C**) and luciferase assay (**D**) using the commercially available luciferase assay kit (Promega). Further, to test whether STAT3C and other oncogenes form a repressor complex with DNMT1 and thereby it inactivates SLC5A8 expression in human breast cancer, we have performed a ChIP assay. We transfected STAT3C and other oncogenes in MCF10A cells and immunoprecipitate with DNMT1 antibody and the resulting DNA was analyzed for the primers designed in SLC5A8 promoter at four different sites. The ChIP1 and 2 primers were designed in exon1 the region where the CpG island located and the ChIP3 and 4 primers were designed away from the CpG island.

Figure 5

Our results again show that constitutively active STAT3 is not involved in SLC5A8 inactivation, even though it involved in transactivation of DNMT1 promoter (Figure 3). However, STAT3C combined with oncogenic HRAS can able to inactivate SLC5A8 expression and form a suppressor complex with DNMT1.

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This report would have benefited from thorough proofreading.

To be considered credible reportable outcomes for the current reporting year abstracts/presentations at scientific meetings must be completed during the specified reporting period.

This was the first year progress report and we did not have enough data to submit an abstract at that time. Moreover, we had a plan to submit our data to some decent journals, like Cell or cancer Cell, and these journals does not like if the data has been presented in the conferences or submitted as an abstract. Thus, we little hesitate to prepare the abstract. Now, we have completed all the proposed work and are preparing two manuscript based on the results obtained from the proposed study and intended to submit the first paper to the Cancer Cell and the title of the paper and authors contribution are listed below.

Molecular Mechanism of SLC5A8 Inactivation in Human Breast Cancer

Selvakumar Elangovan,[1,*] Rajneesh Pathania,[1,*] Sabarish Ramachandran,[1] Sudha Ananth, [1] Ravi Padia,[1] Elangovan Gopal,[1] Nikki Harvel[2], Leslyn Hawthrown[2], Thomas R. Boetter,[3] Sylvia B. Smith,[4] Puttur D. Prasad, [1] Vadivel Ganapathy[1] and Muthusamy Thangaraju[1, 5]

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* These two authors contributed equally in this work

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Running Title: SLC5A8 is a functional target of oncogenic H-Ras in breast cancer

Author contribution: S.E., V.G. and M.T. designed research; S.E., S.R. and M.T. were performed expression analysis, DNMT and HDACs assays, FACS analysis and animal experiments; R.P performed stem cells, mammosphere, tumorosphere and immunofluorescence analyses; S.E. and R.P performed H-Ras-GST pull-down assay; S.E., P. D. P. S.B.S. and M.T. were performed siRNA, promoter and ChIP assays; S.A and E.G were

performed uptake assays; N.K. and L.H. were performed microarray analysis; T.B. generated Slc5a8 knockout mice;
 S.E., V.G. and M.T. analyzed the data and wrote the paper. S.E and R.P. contributed equally in this work and consider as co-first authors.   All authors declare no conflict of interest.

The second paper we intended to submit to the Cell and the title of the paper and authors contribution are listed below.

Slc5a8 inactivation predisposes to early onset of mammary tumorigenesis and accelerated lung metastasis

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* These two authors contributed equally in this work and considered as a co-first authors

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Running Title: SLC5A8 is a potential tumor suppressor for breast cancer

Author contribution: S.R., S.E., V.G. and M.T. designed research; S.E., N.V., S.A., J.P.G. and P.M. performed immunohistochemistry and immunofluorescence experiments; S.E., S.R., P. D. P. and M.T. were responsible for shRNA, IP, IB and promoter and ChIP assays; S.E., D.D.B. and P.V.S. contributed to mouse xenograft studies; S.E., V.G. and M.T. analyzed the data and wrote the paper.

All authors declare no conflict of interest.

All data noted in the report should be represented with an appropriate figure (e.g., functional assays noted in Task 1b).

Now we have shown the functional SLC5A8 functional assay in Figure 4.

There is no indication as to how the data generated in Figure 2B were analyzed and whether the data represented are statistically significant.

As mentioned in the text in Figure 2, we have developed a TetOn inducible SLC5A8 stable expression in MCF7, a human breast cancer cells line. The figure 2a, shows the SLC5A8 expression in three different clones with and without of doxycyclin, a tetracycline analog, (2 µg/ml) and the sodium pyruvate (1mM) for 0, 12, 24, and 48 h. Cells were harvested and processed for the propidium iodide stained cell cycle analysis. The sub G1 populations, SubG0/1, were quantified and plotted (Figure 2b). Values are represented as a Mean and SEM of four independent experiments. Even though there is an apoptosis have seen at 12h, which is not statistically significant. However, at 24 and 48h there is statistically significant apoptosis in these cells.

There is no information provided in boxes 16, 17, or 19a on the Standard Form 298. The PI should ensure that all of the information required on this form is appropriately and completely filled out.

Have completed all sections in the Form 298

CONTRACTUAL ISSUES: Information provided in this first annual report supports the following:

Task 1	Months 1-4	In progress
Task 2	Months 5-8	In progress
Task 3	Months 9-12	In progress
Task 4	Months 13-16	Not yet initiated
Task 5	Months 17-30	Not yet initiated
Task 6	Months 30-36	Not yet initiated

TECHNICAL ISSUES: None.